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13. ABSTRACT (Maximum 200 Words)

Retroviral-based expression libraries have been developed from breast tumor cells, and then screened for cDNAs whose expression confers an invasive phenotype on non-invasive breast tumor cells. Four independent cDNAs have been recovered and restested in these screens (DAP-1, LIPE, HSPA5, ABLIM). The Dap-1 cDNA was orientated in the antisense and is by far the most invasive of the four. When tested in other cell types, only the Dap-1 cDNA exhibited transforming properties. Since Dap-1 has been attributed tumor suppressor properties in other biological systems, it was selected for a more detailed analysis. Anti-sense expression of DAP-1 was associated with activation of the small GTPase RhoA in NIH 3T3 cells, but downregulation of RhoA in MCF-7 cells. No effects were noted for Rac1 or Cdc42, or when DAP-1 was expressed in the sense orientation. Analysis of the actin cytoskeleton of MCF-7 cells in which DAP-1 is suppressed revealed a phenotype that is consistent with loss of RhoA function, and the motile phenotype could be suppressed by expression of activated RhoA. To summarize, we have identified DAP-1 as a tumor suppressor in breast cancer cells that can regulate motility and invasion through regulation of the small GTPase RhoA.

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INTRODUCTION:

We have demonstrated previously that retroviral-based expression screening is a powerful approach for the identification of cDNAs whose overexpression are associated with growth deregulation (1). During the course of these studies, an expression system that was developed by the principle investigator was adapted for the identification of genes that contribute to the progression of breast cancer cells to a metastatic state (2; appended). We have successfully isolated four cDNAs from a MBA-MB-231 derived library (LIPE, DAP-1, abLIM and HSPA5) that can confer a phenotype of increased invasiveness when overexpressed in MCF-7 cells. The most strongly transforming of these cDNAs contains the coding sequences of death activating protein 1 (DAP1) in the antisense orientation. Amongst the four cDNAs, only DAP-1 was able to confer a phenotype of transformation when expressed in other cell types. Interestingly, DAP-1 was originally identified in a screen for positive mediators of apoptosis using antisense cDNA libraries to rescue cells from cell death in the presence of the killing cytokine interferon gamma (IFN-γ)(3). Because DAP-1 exhibited the strongest phenotype of the four new cDNAs (15-fold increase in invasion), and because it has been shown previously to have tumor suppressor activity, it was selected for further analysis. Interestingly, expression of DAP-1 (antisense) in NIH 3T3 cells is associated with activation of the small GTPase RhoA. In contrast down-regulation of RhoA was observed in MCF-7 cells in which DAP-1 expression is suppressed. Changes in the actin cytoskeleton of these cells was observed that were consistent with down-regulation of RhoA activity. No alterations were observed in either RhoA GTP-levels or the actin cytoskeleton when DAP-1 was overexpressed in the sense orientation. Finally, co-expression of DAP-1 (antisense) with an activated derivative of RhoA blocked the ability of DAP-1 to confer a motile phenotype on MCF-7 cells. To summarize, we have identified DAP-1 as a potential tumor suppressor protein that can regulate motility and invasion in human breast cancer cells. This activity appears to be mediated by the ability of DAP-1 to regulate the GTP loading of the small GTPase RhoA.

BODY:

Recovery of proviral inserts from a screen for invasion-inducing proteins. In years 1 and 2 of this proposal we described the construction and initial screening of an MBA-MB-231 derived expression library using MCF-7 cells as recipients. This screen yielded 5 mass populations of cells that were enriched for invasive cells. PCR-based screening of genomic DNAs derived from each cell population identified between 2 to 5 proviral inserts per cell population. Genomic Southerns were performed to confirm the presence of the provirus using a vector-specific probe. Any PCR bands that were confirmed by Southern blot were excised from the gel, recloned into the retroviral vector pCTV3H, and analyzed by restriction digest. A total of 8 clones was recovered which identified 4 distinct cDNA sequences. Additional screens are still ongoing, although no additional cDNAs have yet been identified.

Confirmation of invasive potential of proviral inserts and sequence identification of 4 novel motility inducing cDNAs. The 4 recovered cDNAs were repackaged and used to individually infect MCF-7 cells. Cell populations were selected with hygromycin B, and then tested for motility using a transwell assay. Cognate vector was included as negative control. All four of the cDNAs exhibited increased invasiveness ranging from 2- to 15-fold (Table 1). To summarize, we have identified and retested 4 independent cDNAs, each with invasive potential in MCF-7 cells.

Table 1: Identity and motile phenotype associated with cDNAs recovered from a screen of an MBA-MB-231 derived library for invasion-inducing proteins

Clone Designation	# motile cells / 1 x 10^5 cells plated	Retest Motility?	Clone Identification (genbank accession # / nucleotide residues)
pCTV3H	3 +/- 1		
c1.1	10 +/- 2	++	Hormone Sensitive Lipase (LIPE) (NM005357 / nuc1-3805)
c1.2	67 +/- 12	++++++	DAP-1 antisense (X76105 / nuc 2045-68)
c2.1	13 +/- 4	+	Heat Shock 70kD Protein5 (HSPA5) (NM005347 / nuc5-3461)
c2.2	71 +/- 8	++++++	DAP-1 antisense (X76105 / nuc 2045-68)
c4.1	21 +/- 5	++	Actin Binding LIM protein (ABLIM) (NM006719 / nuc502-6165)
c5.2	83 +/- 13	++++++	DAP-1 antisense (X76105 / nuc 2045-68)

Identity of recovered clones. The identity of the four invasive clones was determined by DNA sequencing (Table 1). All yielded perfect matches to known proteins. These include an actin binding protein (abLIM), a lipase (LIPE), a heat shock protein (HSPA5) and DAP-1. ABLIM is an actin binding protein which is thought to link interactions between actin filaments and cytpolasmic signaling targets (4). LIPE is an 84-kDa cholesterol esterase and triglyceride hydrolase that functions in the release of fatty acids from adipocytes (5). HSPA5 is also known as glucose regulatory protein 78/immunoglobulin heavy chain binding protein (Grp78/BiP) and is a molecular chaperone that is involved in polypeptide translocation across the ER membrane (6). The fourth clone, DAP-1, was isolated three times, and is the only clone that was orientated in the antisense.

The DAP-1 clone was by far the most invasive of the recovered clones (approximately 15-fold over vector). DAP-1 (Death Associated Protein 1) is expressed as a single 2.4kb mRNA that encodes for a basic, proline-rich, cytoplasmic 15kD protein (3). Interestingly, DAP-1 was originally identified in a screen for positive mediators of apoptosis using antisense cDNA libraries to rescue cells from cell death in the presence of the killing cytokine interferon gamma (IFN-γ) (3). Extensive studies have now shown that DAP-1 is phosphorylated by a calcium/calmodulin-dependent DAP kinase which was recently found to have strong tumor suppressive activities, coupling the control of apoptosis to metastasis (7). DAP kinase is localized to actin filaments and this localization is critical for the apoptotic process, specifically to the disruption of stress fibers, a hallmark of apoptosis. Both these genes appear not only to be involved in cell death in response to IFN-γ, but also to apoptosis as a result of detachment from the

extracellular matrix. In the case of our screen we have isolated a 1,977 bp cDNA (nucleotide residues 68-2045) of DAP-1 in the antisense orientation. Therefore, it is presumably through downregulation of DAP-1 that this clone causes an increase in motility. In this way, DAP-1 is acting as a tumor suppressor in that downregulating it causes an increase in invasion capability. .

Expression of DAP-1 (but not LIPE, ABLIM or HSPA5) is associated with transformation in NIH 3T3 cells. Because many oncogenes that affect cell growth also mediate cell motility and invasion, we wondered whether the four invasion inducing cDNAs would have a more generalized transforming activity in NIH 3T3 cells. Towards this end, we carried out NIH 3T3 assays for focus-formation, growth in soft agar, and growth in low serum. The oncogene Dbs was included as a positive control in all assays. We and others have shown previously that Dbs has potent NIH 3T3 cell transforming activity in these three assays (8). Cognate vector was also included in all assays as a negative control. Similar to Dbs, expression of DAP-1 (antisense) in NIH 3T3 cells was associated with enhanced focus-forming activity (Table 2), anchorage independent growth and growth in low serum. In contrast, the transforming activity of LIPE, abLIM and HSPA5 was indistinguishable from the vector controls suggesting that they have a specific function in the context of MCF-7 cells. Interestingly, the morphology of the foci associated with DAP-1 antisense expression were highly reminiscent of those induced by activated derivatives of the Rho family GTPases (data shown in previous report).

Table 2: Transforming activity of invasion-inducing proteins in NIH 3T3 cells.

Identity	Focus-forming Activity (%)[a]	Soft-agar growth (%)[b]	Low serum (%)[c]
Vector	0.1	0.1	0.5
DAP-1	4.3	3.7	11.1
LIPE	0.1	0.1	0.4
ABLIM	0.2	0.1	0.6
HSPA5	0.1	0.1	0.6
Dbs	6.7	5.9	13.7

[a] 10^5 stably transfected cells were mixed with 10^6 untransfected cells and plated onto 10 cm dishes. Foci were counted at 10 days and expressed as percentage of cells plated.
[b] 10^5 stably transfected cells were seeded in 0.5% agar over a base layer of 1%. Colonies were counted at 21 days and expressed as a percentage of cells cultured.
[c] 10^5 stably transfected cells were plated in 10 cm dishes and cultured on 0.5% serum for 14 days. Colonies were counted and expressed as a percentage of cells plated.

Expression of DAP-1 in the antisense is associated with activation of RhoA (but not Rac1 or Cdc42) in NIH 3T3 cells, and inactivation in MCF-7 cells. Since DAP-1 produced a phenotype in NIH 3T3 cells that we have observed previously with activated Rho proteins (8), we wondered whether downregulation of DAP-1 in these cells was associated with increased GTP-loading on Rho. To test this hypothesis, a stable cell line was generated with the DAP-1 antisense clone. Lysates were collected, and then subjected to affinity purification to measure levels of endogenous RhoA, Rac1

and Cdc42. We routinely use these assays to measure the *in vivo* activity of Rho family guanine nucleotide exchange factors (9). Compared to the vector cell lines, we were consistently able to detect elevated levels of RhoA, but not Rac1 or Cdc42 in our stable cell line (Fig. 1a). Thus, downregulation of DAP-1 in NIH 3T3 cells is associated with increased RhoA loading, which probably accounts for its transforming activity.

The ability of antisense DAP-1 to activate RhoA was surprising since we have shown previously that activated RhoA in breast cancer cell lines blocks invasion and motility. To determine whether RhoA activation by DAP-1 occurs in a cell-specific manner, we examined levels of activated RhoA, Rac1 and Cdc42 in MCF-7 cells that stably express DAP-1 in the antisense (Fig. 1b). To further determine whether RhoA-GTP levels in MCF-7 cells respond to DAP-1 expression in a dosage-dependent manner, we also examined cells in which DAP-1 is expressed in the sense orientation. Compared to vector cells (and in contrast to NIH 3T3 cells) we observed reduced levels of activated RhoA in MCF-7 cells in which DAP-1 expression is suppressed. No differences were observed in Rac1-GTP or Cdc42-GTP levels. In contrast overexpression of DAP-1 in the sense orientation had no effect on the levels of activated RhoA, Rac1 or Cdc42 in these cells. We conclude that suppression of DAP-1 levels acts to specifically downregulate RhoA in MCF-7 cells, and that loss of RhoA function may account for the motile phenotype.

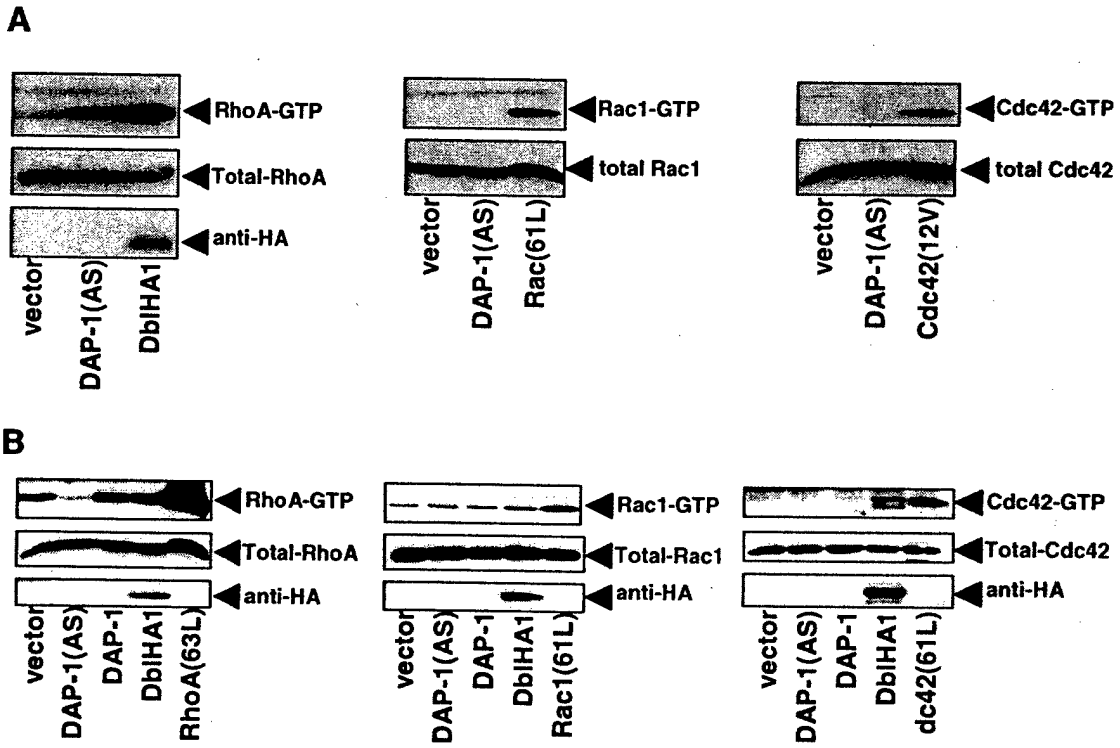

Figure 1: Anti-sense DAP-1 activates RhoA in NIH3T3 cells, but downregulates RhoA in MCF-7 cells. Lysates were collected from (A) NIH 3T3 or (B) MCF-7 cells that stably express the indicated plasmids. DAP-1(AS) = antisense orientation. Lysates were split into three parts, each of which was normalized for expression of either RhoA, Rac1 or Cdc42. Each lysate was then subjected to affinity purification using immobilized GST-Pak (Rac1 or Cdc42) or GST-C21 (RhoA). GTP-bound GTPases were then visualized by Western blot. RhoA(63L), Rac1(61L), and Cdc42(61L) were included as positive controls for RhoA, Rac1, and Cdc42 respectively.

Expression of DAP-1 in the antisense is associated with alterations in the actin cytoskeleton of MCF-7 cells. RhoA has been implicated in multiple cellular activities including entry into cell cycle, regulation of transcription and regulation of the actin cytoskeleton. With respect to the latter, RhoA activation is associated with increased actin stress fiber formation and focal adhesions, both of which should limit cell motility. To determine whether the DAP-1 mediated downregulation of RhoA-GTP in MCF-7 cells is associated with rearrangements of the actin cytoskeleton, we used Phalloidin staining to visualize actin structures in MCF-7 cells that stably express DAP-1 in the antisense (Fig. 2). Again, we also included DAP-1 expressed in the sense orientation as an additional control. When compared to vector controls, the cells that express DAP-1 in the antisense exhibited a dramatic reduction in stress fiber formation which is consistent with inactivation of RhoA. In contrast, no effect was observed in the cells that express DAP-1 in the sense orientation.

Figure 2: MCF-7 cells that express DAP-1 in the antisense have reduced levels of polymerized actin. Cells that stably express either vector, DAP-1 in the antisense (DAP-1(AS)) or Dap-1 in the sense orientation (DAP-1) were plated on coverslips for 48 hr, then fixed and stained for F-actin using phalloidin-rhodamine.

Constitutively activated RhoA blocks the influence of DAP-1 on MCF-7 cell motility. To further examine a role for RhoA in mediating the effect of DAP-1 on cell motility, we asked whether a constitutively activated mutant of RhoA could block the ability of DAP-1 to increase cell motility and invasion. For this analysis we generated an MCF-7 cell line that stably expressed both DAP-1 in the antisense, and the RhoA(63L) mutant. RhoA(63L) is a well characterized, constitutively activated version of RhoA that is locked into the GTP-bound state. This cell line exhibited dramatically reduced motility and invasion when compared to cells that express just the anti-sense DAP-1 alone (Fig. 3). These results are again consistent with our hypothesis that DAP-1 regulates cell motility and invasion by downregulating the activation of RhoA. Work is currently underway to determine the specific RhoA effector proteins that mediate the effects of DAP-1. Candidates that are currently under close scrutiny are the ROCK1and PKN kinases, both of which have been implicated in RhoA-mediated transformation in other cell types.

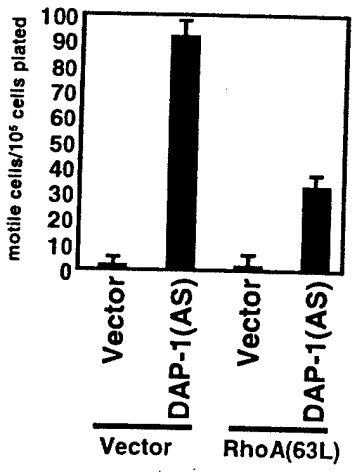

Figure 3: Activated RhoA blocks DAP-1 mediated motility in MCF-7 cells. MCF-7 stable cell lines were generated that expressed the indicated combinations of plasmids. Each cell line was tested for motility by plating 1 x 10^5 cells in the upper chamber of a transwell motility filter. Cells that traversed the filter were stained and counted at 24 hours. Data is expressed as average number of motile cells per filter. All experiments were performed in triplicate using duplicate filters. Data shown is representative of a single assay.

KEY RESEARCH ACCOMPLISHMENTS:

Year 1

- Construction of highly complex cDNA expression libraries from RNA derived from invasive human breast tumor cell lines.
- Establishment of mass populations of human cells that are stably infected with retroviral-based cDNA expression libraries
- Establishment of enrichment protocols that will be required to screen libraries for cDNAs whose expression contribute to an invasive phenotype.

Year 2

- Recovery of proviral inserts from sequentially enriched populations of MCF-7 cells that were stably infected with retroviral particles derived from MBA-MB-231S libraries.
- Identification and complete sequencing of four independent, invasion-inducing cDNAs. One clone identified as the tumor suppressor DAP-1, expressed in the antisense.

Year 3

- Analysis of the transforming potential of all four cDNAs in NIH 3T3 cells.
- Detailed analysis of the transforming and signaling potential of DAP-1 in NIH 3T3 and MCF-7 cells reveals that it has the properties of a general tumor suppressor protein that functions by deregulating the small GTPase RhoA.

REPORTABLE OUTCOMES:

G. M. Mahon and I. P. Whitehead (2001) Retrovirus cDNA expression library screening for oncogenes. Methods enzymol. 332:211-221.

G.M. Mahon and I.P. Whitehead (2002) Expression screening for invasion inducing proteins. Proceedings of DOD Breast Cancer Research Program Meeting, P1-31, Orlando, Fla.

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LIST OF PERSONNEL RECEIVING PAY

Gwendolyn M. Mahon Research Teaching Specialist III

CONCLUSIONS:

The work performed during the course of these studies establishes that our retrovirus-based system can be used to identify cDNAs that result in the increased invasive potential of MCF-7 cells. Thus far four cDNAs has been recovered whose expression cause a phenotype of increased motility and invasion in MCF-7 cells. Although considerable time continues to be spent recovering and retesting proviral inserts from ongoing screens, no additional cDNAs have yet been recovered. Importantly, an extremely low frequency of false positives has been encountered which bodes well for the future of this screening strategy.

A very potent inducer of invasion (an antisense cDNA for DAP-1), was isolated in 3 independent mass populations of motile cells, and was selected for further analysis. We have determined that DAP-1 can function as a tumor suppressor when expressed in other cell types, and that its activity is mediated by its ability to regulate the small GTPase RhoA. Although DAP-1 has been previously attributed tumor suppressor function, its ability to regulate an invasive phenotype through interactions with small GTPases is novel. Further characterization of this molecule and its transforming ability may provide new insights into tumor metastasis. It is anticipated that the results obtained from these studies will form the basis of a more extensive proposal to the NCI.

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APPENDIX:

Manuscripts and Abstracts

G. M. Mahon and I. P. Whitehead (2001) Retrovirus cDNA expression library screening for oncogenes. Methods enzymol. 332:211-221.

G.M. Mahon and I.P. Whitehead (2002) Expression screening for invasion inducing proteins. Proceedings of DOD Breast Cancer Research Program Meeting, P1-31, Orlando, Fla.

EXPRESSION SCREENING FOR INVASION-INDUCING PROTEINS

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Studies aimed at identifying genes whose deregulated expression either contribute to, or accompany, the development of breast cancer have been relatively unsuccessful. This can be largely attributed to the technical difficulties associated with the transfer of large libraries of cDNAs into human breast epithelial cells. Thus, novel oncogenes are still identified using traditional, fibroblast-based transformation assays, even though most prove to be biologically inert when expressed in epithelial cells. Since most human breast tumors are of epithelial origin, it is imperative that these technical limitations be addressed. Here we describe the development of a novel, epithelial-based expression cloning system that can be used to identify genetic sequences whose deregulated expression contribute to breast tumor progression. The general approach to cloning invasion-promoting cDNAs is to make expressible cDNA libraries from highly invasive human breast tumor cell lines, and to transfer these libraries to human tumor cells that normally do not exhibit invasive properties. Cells that acquire an invasive phenotype as a result of the expression of a transferred cDNA are selected out of the population using a transwell-based selection protocol. cDNAs are then recovered by PCR, and sequenced to determine their identity. cDNA library transfer is achieved through the use of retroviral vectors and transient packaging systems, which enables the transfer of complex cDNA libraries and their expression at consistently high levels. Retroviral vectors also permit the use of human breast epithelial cells as recipients, which is critical for identifying proteins that participate in signal transduction pathways that are relevant to epithelial cells. As a preliminary test of this system, we screened an expression library derived from the MBA-MB-231 cell line. MCF-7-EcoR cells were stably infected with retroviral particles derived from the library. Five cell populations (50,000 cells each) were screened for invasive clones. Following three rounds of library enrichment, DNA was isolated and discrete proviral inserts were identified by PCR. Sequence analysis and transforming properties of these inserts will be discussed.

The U.S. Army Medical Research and Materiel Command under DAMD17-00-1-0365 supported this work.

[16] Retrovirus cDNA Expression Library Screening for Oncogenes

By GWENDOLYN M. MAHON and IAN P. WHITEHEAD

Although conventional, plasmid-based, gene transfer assays have been used to identify protooncogenes, only a handful of transforming sequences have been detected in this manner. The limited success of these efforts reflects technical limitations of the systems used, rather than exhaustion of the pool of cDNAs with oncogenic potential. The most severe limitations associated with the use of plasmid-based libraries for expression cloning (low efficiencies of transfection and expression) have now been overcome by the development of retrovirus-based cDNA transfer systems.[1-4] In these systems, cDNA expression libraries are constructed in retroviral plasmids, and then converted into libraries of infectious retroviral particles. Four major advantages are obtained through the use of retroviral library transfer: (1) the ability to screen large numbers of cDNA clones on an equivalent number of recipient cells, (2) the relatively high levels of expression obtained with retrovirally transferred cDNAs, (3) the potential to use cell lines that have been inaccessible to expression cloning because of low transfection efficiencies, and (4) the development of highly efficient recovery mechanisms for the proviral inserts.

We have described a retrovirus-based cDNA expression system and its successful application to the identification of novel oncogenes.[2] This system, which is described here in detail, permits the stable transfer and expression of large numbers of cDNA clones into equivalent numbers of recipient cells. This allows for the efficient screening of complex cDNA libraries, and facilitates the identification of transforming sequences that are present at low frequency within the cDNA population. Although we are describing methods that identify transforming cDNAs on the basis of their focus-forming activity,[5] this expression system can be readily adapted for use in alternative screens for growth transformation.

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Construction of Retrovirus-Based cDNA Expression Libraries

Retroviral Vectors

We have described the construction of a retroviral vector (pCTV3B) that has been designed specifically for use in cDNA expression cloning (Fig. 1).[2] The vector has been made as compact as possible (~5560 bp) to maximize the stability of cDNAs, and all splice sites have been removed so that they cannot activate cryptic splice sites that may be present within the cDNA inserts. In addition to viral sequences, the viral transcripts that are derived from pCTV3B contain an inserted cDNA, a bacterial selectable

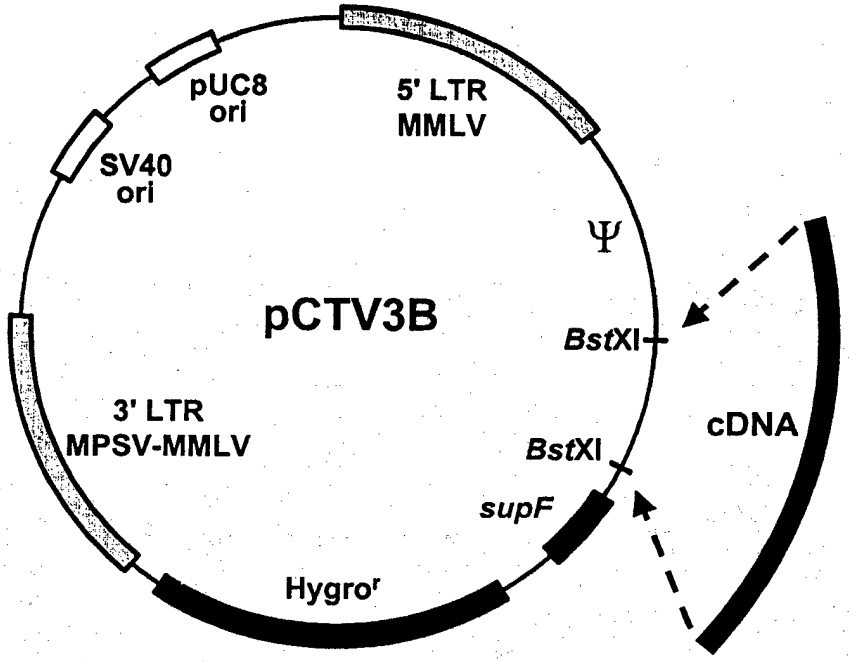

FIG. 1. Structure of the pCTV3B retrovirus-based expression cloning vector. pCTV3B consists of (1) a 5′ Mo-MuLV (MMLV) LTR with an extended *gag* region (Ψ) that lacks the normal initiation codon, (2) a cDNA cloning site consisting of two *Bst*XI sites separated by a 400-bp stuffer fragment, (3) a *supF* gene that provides a bacterial selectable marker, (4) the hygromycin phosphohydrolase gene, which provides a mammalian selectable marker (Hygro^r), (5) a composite MPSV/MoMuLV 3′ LTR, (6) the replication origin from pUC8 to facilitate propagation in bacterial cells, and (7) the simian virus 40 (SV40) origin of replication. After viral transmission, the region between (and including) the 5′ and 3′ LTRs becomes stably integrated into the host genome as a proviral insert. Both the 5′ and 3′ LTRs of the provirus are derived from the composite MPSV/Mo-MuLV 3′ LTR.

marker (*supF*),[6] and a marker that permits selection in mammalian cells (Hygro^r).[7] An extended *gag* region ensures efficient packaging of this viral transcript when it is expressed in an appropriate packaging cell line.[8]

The pCTV retroviral vectors utilize the *supF*–p3 selection system for *Escherichia coli* that was developed by B. Seed.[9] The *supF* gene encodes a tRNA molecule that can rescue amber mutations within antibiotic resistance genes, and thus provides a compact (~220 bp) bacterial selectable marker for the retroviral vectors. The p3 plasmid is a 50-kbp, single-copy, stably propagated plasmid that encodes a wild-type kanamycin resistance (Kan^r) gene as well as amber mutant ampicillin and tetracycline resistance genes. *Escherichia coli* strains that contain p3 (e.g., MC1061/p3[10]) can be used to select for plasmids that are carrying *supF*.

Isolation of Poly(A) mRNA

The successful construction of a complex library (>10^6 clones) generally requires 3–5 μg of good quality poly(A) mRNA. Such quantities can be readily obtained from tumor-derived, mammalian cell lines. Total RNA is first isolated with Trizol reagent (GIBCO-BRL, Gaithersburg, MD) according to the manufacturer instructions. We collect lysates from at least 2×10^8 cells (e.g., 20 confluent 100-mm dishes), which will yield in excess of 1 mg of total RNA. The RNA is then subjected to two rounds of purification on oligo(dT)-cellulose columns to obtain 10–50 μg of purified poly(A) mRNA.[11] Because we generally use random priming during cDNA synthesis, it is important to carry out the second round of purification to remove as much ribosomal RNA as possible.

Preparation of cDNAs

When constructing cDNA libraries that are to be screened for oncogenes, we find it advantageous to use random priming and bidirectional cloning. The majority of oncogenes that have been described, to date, are activated by truncation, and random priming enriches the library for truncated cDNAs. Bidirectional cloning allows us to simultaneously screen for oncogenes and tumor suppressors. The protocol that we are describing generates blunt-ended cDNAs that can be fused to *Bst*XI linkers, and then

[6] B. Seed, *Nucleic Acids Res.* **11**, 2427 (1983).
[7] H. U. Bernard, G. Krammer, and W. G. Rowekamp, *Exp. Cell. Res.* **158**, 237 (1985).
[8] M. A. Bender, T. D. Palmer, R. E. Gelinas, and A. D. Miller, *J. Virol.* **61**, 1639 (1987).
[9] B. Seed, *Nature* (*London*) **329**, 840 (1987).
[10] B. Seed and A. Aruffo, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 3365 (1987).
[11] T. Maniatis, E. F. Fritsch, and J. Sambrook, "Molecular Cloning: A Laboratory Manual." Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989.

cloned into the retroviral pCTV3B vector. We use SuperScript II reverse transcriptase (GIBCO-BRL) for first-strand synthesis. Second-strand synthesis is carried out by nick translation using *E. coli* DNA polymerase I, *E. coli* RNase H, and *E. coli* DNA ligase (all from GIBCO-BRL).

All glassware and solutions used for first-strand synthesis must be RNase free. For each reaction it is preferable to work with at least 3–5 μg of good quality mRNA that has been resuspended in RNase-free Milli-Q (Millipore, Danvers, MA) water. This should generate sufficient quantities of cDNA to allow size fractionation of the libraries. In a sterile 1.5-ml tube combine 4 μl of a 50-ng/μl random hexamer mix 5 μg of mRNA, and RNase-free water to a final volume of 21 μl. Incubate at 70° for 10 min, and then chill on ice. Add 8 μl of 5× first-strand buffer (GIBCO-BRL), 4 μl of 0.1 *M* dithiothreitol (DTT), and 2 μl of a 10 m*M* dNTP mix. Mix the contents gently by vortexing, and incubate for 2 min at 37°. Add 5 μl of SuperScript II (200 U/μl), mix gently, and continue the incubation (37°) for 1 hr. Place the mixture on ice and proceed with second-strand synthesis.

To the first-strand reaction mixture add the following, in order (on ice): 71 μl of distilled H_2O, 30 μl of 5× second-strand buffer (GIBCO-BRL), 3 μl 10 m*M* dNTP mix, 1 μl (10 U) of *E. coli* DNA ligase, 4 μl (40 U) of *E. coli* DNA polymerase I, and 1 μl (2 U) of *E. coli* RNase H. Mix by gently vortexing, and incubate at 16° for 2 hr. Then, add 2 μl (10 U) of T4 DNA polymerase and incubate at 16° for a further 5 min. Place on ice and stop the reaction with 10 μl of 0.5 *M* EDTA. Extract with 150 μl of phenol–chloroform, and precipitate with 70 μl of 7.5 *M* ammonium acetate and 500 μl of ice-cold, 100% ethanol. Centrifuge at 14,000*g* for 20 min at 4°, wash the pellet with ice-cold 70% (v/v) ethanol, and resuspend in 20 μl of distilled H_2O. Load an aliquot (2 μl) on a 1.2% (w/v) agarose gel to check the quality and size range of the cDNAs.

Linkering of cDNAs

The *Bst*XI linkering system that has been adapted for use with the pCTV vectors has been described previously.[9] The *Bst*XI linkers substantially decrease the number of transformants that do not contain an insert, and reduce the possibility of having two cDNAs ligated into the same vector. These adapters have also been designed to introduce stop codons in all three reading frames at the 3′ end of the cDNA, thus preventing translational readthrough into the vector sequences. Oligonucleotides should be synthesized with 5′-phosphates or should be kinased prior to annealing (13-mer, 5′-TCA GTT ACT CAG G; 17-mer, 5′-CCT GAG TAA CTG ACA CA). To anneal oligonucleotide pairs, combine equimolar amounts of each oligonucleotide, heat to 60°, and then allow to slowly cool

to room temperature. Ligation reactions consist of 1–5 μg of cDNA, 0.5 μg of linkers, 4 μl of 5× ligation buffer, 2 U of T4 DNA ligase, and water to 20 μl. Incubate the ligation reactions at 16° for 4–8 hr.

Size Fractionation of cDNA

To avoid the selection against large cDNAs that often occurs during library construction, we have found it beneficial to separate our linkered cDNAs into several size classes prior to ligation into the retroviral vector. This facilitates the separate transformation of larger cDNAs and affords the opportunity to adjust their representation in the final libraries. Load the cDNA linkering reaction onto a 1.2% (w/v) agarose gel (containing ethidium bromide) with flanking size markers. Run the cDNA a sufficient distance into the gel to obtain reasonable marker separation while still minimizing the size of the gel slice. Slice off the flanking marker lanes, expose them to UV, and mark off the appropriate size ranges. Do not expose the cDNA lane to UV. We normally isolate fractions with size ranges of 1000 to 2500 bp and 2500+ bp. Realign the marker lanes with the cDNA lane and excise the appropriately sized gel segments. Purify the cDNA by the GeneClean procedure (Bio 101, La Jolla, CA). This provides reasonable yields of cDNA while eliminating unligated adapters.

Ligation of Linkered cDNA

The cloning site of the pCTV3B retroviral vector consists of two *Bst*XI sites separated by a 400-bp stuffer fragment (Fig. 1).[11] Complete digestion of the vector with *Bst*XI removes the stuffer and generates two noncomplementary ends that can be ligated to the *Bst*XI-linkered cDNAs. The retroviral vector is digested to completion with *Bst*XI, dephosphorylated, and purified on a 1.2% (w/v) agarose gel followed by electroelution and precipitation. As with the cDNA, it is important not to expose the vector DNA to UV at any time. Ligation reactions consist of 20 ng of vector, an equimolar amount of cDNA, 1 μl of 10× ligation buffer [250 m*M* Tris-HCl (pH 7.8), 50 m*M* $MgCl_2$, 10 m*M* DTT, 10 m*M* ATP], H_2O to 10 μl total volume, and 0.5 U of T4 Ligase. This ligation reaction includes a minimal ionic strength ligation buffer that is required specifically for electroporation. Incubate the ligation at room temperature for 3 hr, and then add 10 μl of H_2O and heat kill at 72° for 20 min.

Generating Retroviral Plasmid Libraries

Libraries of retroviral plasmids are generated by electrotransformation of MC1061/p310 *E. coli* [we use a Bio-Rad (Hercules, CA) Gene Pulser

II].[10] The transformation mixes are plated in soft agar, which substantially reduces the variation in colony size and allows large numbers of colonies to be plated (up to 750,000) on single 15-cm plates. Because the size of the library is determined, in part, by the success of the electroporation, we use only electrocompetent MC1061/p3 cells that have a competence greater than 5×10^9.

Thaw electrocompetent MC1061/p3 *E. coli* on ice until completely melted. Add up to 1.5 μl of the ligation mix to a prechilled tube, and then add and gently mix 40 μl of electrocompetent cells. Allow to sit for 1 min on ice and then transfer to a prechilled 1-mm cuvette. Electroporate the cells (1600 V, 25 μF, and 200 Ω) and then immediately add 1 ml of YT broth (with 20 m*M* glucose) to the cuvette.[11] Transfer the cells to a new tube, incubate in a 37° water bath for 10 min, and then shake at 37° for an additional 110 min. Bring the volume of the cell solution up to 5 ml with more YT and warm to 37° in a water bath. Put 5 ml of melted 1.2% (w/v) agar–YT containing ampicillin (100 μg/ml) and tetracycline (15 μg/ml) in a second tube and incubate in a 44° water bath. Once both tubes are temperature equilibrated, combine the two tubes, mix by inversion, and quickly pour on the surface of a 15-cm plate of 1.2% (w/v) agar–YT [ampicillin (50 μg/ml)–tetracycline (7.5 μg/ml)]. Let the soft agar set at room temperature for 15 min and then incubate at 37° for 20 hr. The library size can now be determined by counting the number of colonies on the plates.

Recovering Plasmid Library from Soft Agar

Once the plasmid library has been grown in soft agar, we recommend that the cells be harvested, and the DNA isolated, within 24 hr. Plates can be stored at 4° prior to harvesting. Gently wash the surface of the plate with YT to remove most of the large surface colonies. Scrape off the soft agar into 50-ml Falcon tubes, mash it well, and then transfer it to a 50-ml syringe. Add 10 ml of YT to the slurry and run it three times through an 18-gauge needle and then three times through a 22-gauge needle. Mix in 10 ml of Sephadex G-25 (medium; Sigma, St. Louis, MO) that has been autoclaved in YT (G-25/YT). Prepare spin columns (one per plate) from 20-ml syringe barrels, plugged with a wad of glass wool and resting in 50-ml Falcon tubes. Add a 10-ml bed of G-25/YT to each column and then overlay with the library/G-25/YT mix. Centrifuge the columns at 500 rpm in a swinging bucket rotor for 5 min at room temperature, and then increase to 1000 rpm for an additional 10 min. Centrifuge the eluted medium at 4000*g* for 10 min at room temperature to recover the cells, and then resuspend the pellet in 10 volumes of YT. To prepare a glycerol stock, add 0.5 ml of the cells to 0.5 ml of YT–40% (v/v) glycerol, freeze in a dry ice–ethanol bath for 10 min, and then store at −70°. To prepare a library of plasmid DNA, dilute the remaining cells to an A_{600} of 0.1 in YT, and then grow to an A_{600} of 1.5. DNA can now be prepared by alkaline lysis,[12] followed by purification through ethidium bromide–cesium chloride gradients.[11]

Converting Plasmid Libraries to Viral Libraries

Packaging cells are mammalian cell lines that stably express all the viral components that are necessary to recognize and package viral mRNAs. Such cell lines can facilitate the simultaneous and proportional conversion of a plasmid library into a library of retroviral particles. When pooled plasmid DNAs from the cDNA library are introduced into these cell lines, viral mRNAs (including the inserted cDNA) are transcribed, packaged into infectious retroviral particles, and released into the medium. These particles can then be harvested and either stored or used for screening. Packaging cell lines vary in the viral titers that can be obtained. We have found that the high titers ($>10^6$/ml) that can be routinely obtained with the BOSC23 cell line are more than sufficient to ensure the efficient screening of highly complex libraries.[13]

Pooled plasmid DNAs from the cDNA libraries are introduced into the BOSC23 cells by calcium phosphate transfection. Unlike most adherent cell lines, BOSC23 cells do not form even monolayers and will begin clumping before confluence is reached. To ensure a high efficiency of transfection, it is important that this clumping be minimized. To achieve this, we begin splitting the cells 1:1, 2–3 days prior to transfection. If this is repeated on two or three consecutive days, it is possible to obtain high-density plates of well-spread cells. These cells can be readily trypsinized, and can be accurately counted and plated.

Plate 2×10^6 BOSC23 cells per plate (60 mm), 24 hr prior to transfection. Optimal transfection density is about 80% confluence. Be precise—relatively small variations in cell density, either high or low, can sometimes have dramatic effects on transfection efficiencies and/or cell survival. Prior to transfection, change the medium to 4 ml of Dulbecco's modified Eagle's medium (DMEM)–10% (v/v) fetal bovine serum (FBS) containing 25 μM choloroquine. Transfect by adding 10 μg of DNA to 500 μl of HEPES-buffered saline (pH 7.05),[11] and then add (while vortexing) 50 μl of 1.25 *M* $CaCl_2$. Immediately add this solution to the cells. At 10 hr, replace the medium with 4 ml of DMEM–10% (v/v) FBS. It is important to remove

[12] H. C. Birnboim and J. Doly, *Nucleic Acids Res.* **7**, 1513 (1979).
[13] W. S. Pear, G. P. Nolan, M. L. Scott, and D. Baltimore, *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8392 (1993).

the chloroquine in this timely fashion to prevent cell killing. We change the volume of the medium to 2.5 ml (fresh medium) at 36 hr posttransfection and then collect the medium 24 hr later. The virus-containing medium should then be filtered (45-μm pore size filter) to remove cells. The virus can now be frozen at −80°, without any significant loss of titer, or used immediately for infections.

Screening Retrovirus-Based Expression Libraries for Oncogenes

Infection of Recipient Cell Lines

We generally use the focus formation assay as the basis of our screens for transforming cDNAs.[5] These screens are performed in adherent, murine cell lines such as NIH 3T3 (fibroblasts) or C127 (epithelial), which are readily infectable by ecotropic virus, and which are susceptible to single-hit transformation. If screens are to be conducted with human cell lines as recipients it would be necessary to use amphotropic packaging cell lines to generate infectious virus.

Plate 2×10^5 cells/plate (100 mm), 24 hr prior to infection. Immediately prior to infection, dilute the viral soup 1:1 in DMEM that contains 10% calf serum (CS) and Polybrene (16 μg/ml; final concentration, 8 μg/ml). Aspirate the medium from the cells and replace with the medium containing virus and Polybrene. Allow the infection to proceed for 3–5 hr and then replace the medium with fresh DMEM–10% (v/v) CS. Change the medium every 2–3 days for up to 21 days and identify foci that are formed. We do not perform these screens under selective conditions because the infection frequency is usually high, and selection often disrupts the integrity of the monolayer, thus generating spontaneous foci. Individual foci are then scraped from the surface of the plate, transferred to individual plates (35 mm), and amplified clonally. Clonal amplification is done in the presence of hygromycin (200 μg/ml) to ensure the presence of a proviral insert. To determine viral titer, we remove a small aliquot of the viral soup, perform serial dilutions, and then infect recipient cells, under selection, to determine the number of resistant colonies that can be generated.

Polymerase Chain Reaction Amplification of Proviral cDNA Inserts

A polymerase chain reaction (PCR)-mediated DNA amplification protocol has been developed to facilitate the recovery of proviral cDNA inserts (Fig. 2).[2] High molecular weight DNA, prepared from the transformed cells, is PCR amplified with a set of vector primers that flank the cDNA

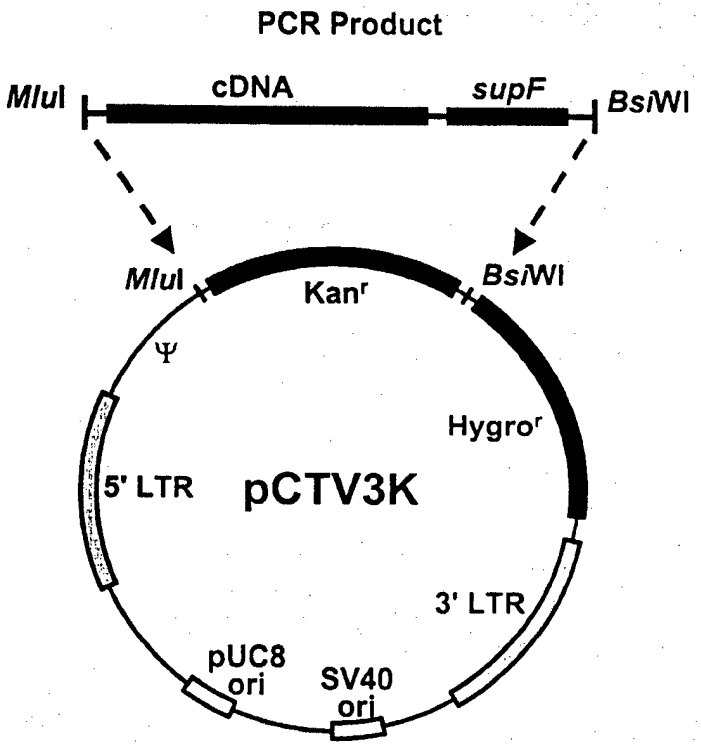

FIG. 2. Procedure for recovering transforming cDNAs from proviral inserts. Genomic DNA is isolated from transformed cell clones and then used as a template for PCR amplification, using a set of vector primers that flank the cDNA insert and the linked *supF* gene. The amplified fragment is cut with *Mlu*I and *Bsi*WI and cloned into complementary sites within the pCTV3K vector. Recombinants are isolated on the basis of the acquisition of the suppressor tRNA activity (*supF*).

insert and the adjacent *supF* gene. *supF* encodes suppressor tRNA activity and can be used as a selectable marker in bacterial strains that contain the p3 plasmid. PCR products that contain the *supF* sequences are then cloned into a retroviral vector that lacks a bacterial selectable marker and selected on the basis of their acquired suppressor tRNA activity. The PCR process utilizes *Pfu* DNA polymerase (Stratagene, La Jolla, CA), which, unlike more common versions of *Taq*, has a proofreading function associated with it. Thus, errors introduced into the DNA sequences by the polymerase are kept to a minimum.

Add 600 μl of lysis buffer [1 m*M* EDTA, 1% (w/v) SDS, and proteinase K (100 μg/ml) added directly before use] to a 35-mm well of confluent cells and incubate at 37° for 90 min. Transfer the lysate to a 1.5-ml tube

and extract twice with an equal volume of phenol–chloroform. Precipitate the DNA twice with 1/10 volume of 10 *M* ammonium acetate and 2 volumes of 95% (v/v) ethanol. Wash thoroughly with 70% (v/v) ethanol after each precipitation. Dry the pellet and resuspend in 150 μl of distilled H_2O. Prepare PCRs containing 100 ng of genomic DNA template, 100 ng of each primer, 2.5 μl of dNTP mix at 2 m*M* each, 2.5 μl of *Pfu* polymerase 10× buffer, 2.5 U of *Pfu* DNA polymerase, and water to 25 μl. We use the following primers for amplifying pCTV3-derived proviruses: pCTV-5′ CCT CAC TCC TTC TCT AGC TC and pCTV-3′-TCG AAT CAA GCT TAT CGA TAC G. PCR cycles are 95° for 60 sec, 50° for 30 sec, 68° for 6 min; for 30 cycles. At this point we run 5 μl of the PCR reaction mix on a 1.2% (w/v) polyacrylamide gel to identify any bands that have been amplified (often there is more than one). We then do a Southern blot with a probe specific for the *supF* gene. This allows us to distinguish bands that are derived from bona fide proviral inserts, and allows us to identify amplified products that are present in subvisual amounts.

Cloning Polymerase Chain Reaction Products

pCTV3K is a specialized retroviral vector that has been developed for use in cloning PCR-amplified proviral inserts (Fig. 2).[2] The vector is derived from pCTV3B and contains a Kan^r marker in place of *supF*. Ligations are performed by replacing the Kan^r marker of pCTV3K with the amplified transforming cDNA and its linked *supF* gene. Because pCTV3K does not contain any ampicillin or tetracycline resistance, recombinants can be recovered in p3 hosts with no background derived from religated vector.

Bring the volume of the PCR to 180 μl with water and then add 20 μl of 10 *M* ammonium acetate. Extract once with 200 μl of phenol–chloroform and then precipitate with 2 volumes 95% (v/v) ethanol. Centrifuge for 30 min at low temperature (15°) and rinse the pellet with 70% (v/v) ethanol. Dissolve the pellet in water, and digest with 10 U each of *Mlu*I (37°) and *Bsi*WI (55°). Run the digests on a 1.2% (w/v) agarose gel and purify the proviral fragments (determined by the Southern blot) by electroelution and ethanol precipitation. Combine 20 ng of the PCR fragment with 40 ng of pCTV3K that has been digested with *Mlu*I and *Bsi*WI, and ligate for 1 hr at room temperature. Use the ligation mix to transform chemically competent (>5 × 10^6) MC1061/p3. Plasmids can be prepared by an alkaline lysis procedure,[12] followed by RNase digestion. The plasmid DNA that is recovered is a fully reconstituted retroviral vector that can be used for functional testing of the transforming cDNA.

Concluding Remarks

It is our experience that these screens most often fail because of a lack of diligence in the cDNA recovery process. Because of the high efficiencies associated with retroviral infections, it is not uncommon to obtain multiple proviral inserts in a single transformed cell clone. Although the smaller, more abundant PCR products are easiest to recover, it is often the larger, subvisual bands that are transforming. Such bands, if identified by Southern blot, can be readily cloned into pCTV3K. On occasion, it is not possible to recover a transforming cDNA from a transformed clone. This will occur if transformation is due to a spontaneous event, insertional mutagenesis, or the combined expression of two or more cDNAs. Because the system has not been designed to characterize such transforming events, we have generally found it prudent to set these clones aside. If the screens are performed correctly, and carefully, a large proportion of transformed cell clones will yield transforming cDNAs.

[17] Identification of Ras-Regulated Genes by Representational Difference Analysis

By JANIEL M. SHIELDS, CHANNING J. DER, and SCOTT POWERS

Introduction

ras genes are among the most frequently mutated oncogenes found in human cancers.[1,2] Since its discovery in 1982 as a transforming oncogene, there have been numerous studies to understand the function of Ras and the pathways utilized by Ras to mediate its actions. To date, most of the studies have focused on the identification of the Ras effector proteins and analyses of the various signaling pathways activated. In contrast, while great strides have been achieved in understanding signaling from the plasma membrane to the nucleus, relatively little is known about which genes are the targets of these signals. Ras signaling pathways lead to the activation of a variety of transcription factors including the Ets family (Elk-1, Ets1, Ets2), Jun, ATF-2 (activating transcription factor 2), Fos, NF-κB, and SRF (serum response factor).[3] Consequently, Ras can stimulate transcription from promoters that contain AP-1 (Fos : Jun, Jun : Jun, Jun : ATF-2 dimers),

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